

b.3) Specific T cell manipulation (induction or blocking). The peptide-MHC I complexes disclosed here will be able to interact with the T cell receptor of a given target cell. In order to stimulate the cell its T cell receptors must be cross-linked. Thus, one can expect that polymerised peptide-MHC I complexes (say "tetramers") might stimulate appropriate peptide-specific, MHC I-restricted T cells, whereas soluble peptide-MHC I complexes might block the same cells.

b.4) Monitoring the specific effect of immune manipulations (e.g. vaccinology). Any investigation on how to manipulate the T cell immune system will - until further improvements in technology - depend on the existence of the "tetramer" (or tetramer-like) technology. The ability to induce specific predetermined responses can be accurately and easily determined, and by modern FACS analysis even further analysed into which subpopulations are affected and how much. The tetramer technology will therefore be essential for future developments in immune manipulations

15

b.5) Purifying specific T cells. Tetramers should allow the efficient purification of peptide-specific, MHC I-restricted T cells. By way of example, if the tetramers through their biotin were coupled to paramagnetic beads it would be relatively straightforward to purify the corresponding specific T cells using a magnet. The specific T cells could then be eluted off and used for analysis or expanded and used for further immune manipulations (e.g. adoptive T cell transfer). This would constitute a vast improvement compared to present cloning technology which is extremely cumbersome and slow.

c) Therapy

25

c.1) Vaccine development. The effects on vaccine development can be crudely subdivided into a direct and an indirect effect. A direct effect of the disclosed technology would be the therapeutic application of the principle mentioned in b3, where a highly specific and very efficient activation of specific T cells (and subsequently of the immune system) is envisioned based on isolated, peptide loaded MHC I molecules administered in a stimulatory way (cross-linked as "tetramers", on beads etc.). An indirect effect would be caused by the improved identification of vaccine candidates that will be the result of the disclosed technology. By enabling a large scale detailed analysis of all human MHC I molecules it will improve predictions of pathogen derived peptides capable of binding to MHC, and allow an easy validation of such predictions (MHC specific epitope analysis).

35

c.2) Treatment of autoimmune diseases. The effects on the treatment of autoimmune diseases can be crudely subdivided into a direct and an indirect effect. A direct effect of the disclosed technology would be the therapeutic application of the principle mentioned in
5 b3, where a highly specific blocking of specific T cells (and subsequently of significant parts of the immune system) is envisioned based on isolated, peptide loaded MHC I molecules administered in a non-stimulatory way (i.e. as soluble, non-cross-linked complexes). An indirect effect would be caused by the improved identification of autoimmune disease inducing candidates that will be the result of the disclosed technology. By ena-
10 bling a large scale detailed analysis of all human MHC I molecules it will improve predictions of self-protein derived peptides capable of binding to MHC, and allow an easy validation of such predictions.

c.3) Purification of T cells for adoptive transfer. As detailed in b5. T cells can be specifically labelled using "tetramers" - and therefore also sorted (i.e. by magnetic beads) leading to the preparation of pure T cell populations of predetermined specificity. Such T cell populations can then be expanded and used e.g. for adoptive transfer against infectious or oncogenic diseases.

20 c.4) Treatment of cancer. Many cancers are associated with mutated oncogenes/tumor suppressor genes, or with dysregulated oncogenes/suppressor genes. The resulting change in the cellular metabolism can be detected by the immune system. There are now examples of dysregulations leading to an altered level of an otherwise completely normal self protein. Yet, the altered level of the self-protein renders it immunogenic and tumor-
25 rejection has been demonstrated in such cases (Vierboom et al., 1997). Mutations and dysregulation can be detected as exemplified, but not limited to, by genetic analysis (e.g. "single strand conformational polymorphism", "selective polymerase chain reaction" or "differential display") or protein analysis (mass spectrometry driven proteome analysis). Any such identified protein can be subjected to the above described MHC specific epitope
30 analysis and specific treatment of the cancer can be attempted as detailed in c1 and c3.

LEGEND TO FIGURES**Figure 1:**

5 Production of recombinant HLA-A*0201. XA90 cells encoding truncated A2 (1-275) was induced with 0.4 mM IPTG. Production was analysed in 15% SDS-PAGE at reducing conditions.

Lane 1: markers as indicated. Lane 2: cellular proteins before induction. Lane 3: cellular proteins 3 hours after induction. Lane 4: recombinant HLA-A*0201 after purification using
10 anion-exchange chromatography.

Figure 2:

Anion-exchanged fractions with recombinant HLA-A*0201 heavy chain from urea solubi-
15 lised inclusion body proteins analysed in a 15% SDS-PAGE at non-reducing condition. Fractions corresponding to purified peptide binding monomers (fraction numbers indicated at the top, compare with figure 4) were analysed. It is shown that recombinant HLA-A*0201 migrates as two distinct proteins about 31-32 kD; protein band 2a and 3, both with intact disulfide bonds.

20

Figure 3:

SDS-page analysis of purified recombinant HLA-A*0201 molecules in presence or absence of DTT. Lane 1: Highly purified and functional HLA-A*0201 heavy chain resulting
25 from a folding by dilution process (non-reduced). Lane 2 and 3: Anion exchanged HLA-A*0201 heavy chain at non-reducing condition (band 2a and 3). In lane 3 it is shown that both proteins bands are partly reduced to band 1 by the reductant present in lane 4. Lane 4: Anion exchanged HLA-A*0201 heavy chain at reducing condition revealing protein band 1, which migrates slower than protein band 2a and 3.

30

Figure 4:

Anion exchanged fractions analysed for amount of eluted proteins and corresponding capacity of peptide binding. The concentrations were determined by BCA and measured at
35 OD562 (full line). The same fractions were also tested for peptide binding. About 30% of

the total amount of protein applied to the column do not bind (basic or neutral charged proteins). These proteins correspond to the bacterial background proteins co-purified with the inclusion bodies. Recombinant HLA-A*0201 molecules eluted with about 200 mM NaCl and were identified by peptide binding analysis (stippled line) and SDS-PAGE analysis (figure 3).

Figure 5:

Immunoprecipitation of folded recombinant HLA-A*0201 molecules using specific antibodies.

Complexes of recombinant HLA-A*0201 heavy chain, b2m and radiolabelled peptide were incubated with monoclonal antibodies against either HLA-A*0201, H-2K^{*} or H-2D^b molecules at 4°C. Subsequently, protein A was added to precipitate immune complexes. The precipitate was washed repeatedly and counted for radioactivity. Only HLA-A*0201 specific antibodies BB7.2 and W6/32 could precipitate the recombinant HLA-A*0201 bound radiolabelled peptide. There was no measurable interaction between recombinant HLA-A*0201 and antibodies with irrelevant specificity.

Figure 6:

Peptide binding to recombinant HLA-A*0201 heavy chain.

Increasing doses of recombinant HLA-A*0201 heavy chains were incubated with b2m (1 µM) and tracer amount of radiolabelled peptide (2 nM) for 4 hr. at 18°C. The degree of complex formation was determined by G25 spun-column chromatography.

Figure 7:

The affinity of peptide interaction with recombinant HLA-A*0201.

5 nM recombinant HLA-A*0201 was incubated with trace amounts of radiolabelled peptide and b2m (1 µM) and increasing concentrations of unlabeled peptides with specificity to HLA-A*0201 and H2-Kk, respectively. The reactions were incubated for 4 hr. at 18°C, and the degree of complex formation was determined by G25 spun-column chromatography.

The bound ligand concentration vs. the ratio of bound and free ligand was plotted to obtain a Scatchard plot (insert).

Figure 8:

5

The b2m dependent peptide binding to recombinant HLA-A*0201.

Recombinant HLA-A*0201 heavy chains were incubated with tracer amount peptide and increasing concentrations of human and mouse b2m as indicated. The reactions were incubated for 4 hr. at 18°C, and the degree of complex formation was determined by G25 spun-column chromatography.

Figure 9:

15 Increasing doses of recombinant HLA-A*0201 heavy chains were incubated with radio-labelled human b2m in presence or absence of specific peptides (10 uM) for 4 hr. at 18°C. The reactions were incubated for 4 hr. at 18°C, and the degree of complex formation was determined by G50 spun-column chromatography.

Figure 10:

The dissociation of b2m from recombinant HLA-A*0201 complex. Spun column purified complexes of heavy chain and radiolabelled b2m with or without peptide were mixed with 3 uM unlabeled b2m and incubated for the time indicated at 4°C. The degree of dissociation was determined by Sephadex G50 spun column chromatography.

Figure 11:

Tetramer staining as assessed by FACS analysis. The cells analysed are CD8 positive T cells either from mice carrying a transgene for a T cell receptor specific for the KAVYN-FATM peptide in association with the H-2D^b or from non-transgene control H-2D^b mice. The analysis was performed with phycoerythrin-streptavidin generated tetramers involving either the relevant KAVYNFATM peptide in complex with recombinant H-2D^b and biotinylated b2m, or with the irrelevant FAPGNYPAL peptide in complex with recombinant H-2D^b and biotinylated b2m. The FACS analysis was performed with tetramer staining on

the x-axis and the CD8 staining on the y-axis. The amount of tetramer positive, CD8 positive cells are seen in the upper right quadrangle. The percentage of total cells being both tetramer and CD8 positive is calculated to the right of the frame and is given directly in the upper right quadrangle.

5		T cells	Complexes	% tetramer+ and CD8+
	Fig. 11A	relevant	relevant	53%
	Fig. 11B	relevant	irrelevant	2%
	Fig. 11C	irrelevant	relevant	1%
10	Fig. 11D	irrelevant	irrelevant	3%

EXAMPLES

15 Materials and methods

Urea, phenylmethylsulfonyl fluoride (PMSF), isopropyl-b-D thiogalactidase (IPTG), bicinchoninic acid solution (BCA) and tris[hydroxymethyl]aminomethane (tris) were purchased from Sigma. Sephadex G50 and Q-sepharose fast flow anion-exchange material were

20 purchased from Pharmacia, Sweden

Production of human and murine MHC class I heavy chains.

Recombinant HLA-A*0201 heavy chain (1-275) in XA90 cells was a kind gift from Drs. Wiley and Garboczi. XA90 cells from an over night culture were inoculated and grown at 25 37°C for production in 200 ml Luria-Bertani medium in 100 µg/ml ampicillin.

Recombinant H-2D^b (1-276) in pGMT7 vector (a pET derivative) was a kind gift from Dr Gallimore. The H-2D^b containing plasmid was transformed into Escherichia coli strain BL21(DE3) (Novagen) and grown at 37°C in 200 ml Luria-Bertani medium containing 100 µg/ml ampicillin. The cells from an over night culture were inoculated and grown at 37°C 30 in 200 ml Luria-Bertani medium in 100 µg/ml ampicillin for production.

Protein expression was induced at midlog phase (A₆₀₀ = 0.6) with 0.4 mM isopropyl-b-d-thiogalactosidase (IPTG). The cells were harvested by centrifugation after 3 hours. The

cells were suspended in 10 ml. 20 mM tris buffer, pH 8.0 and 1 mM EDTA. and stored at -20°C.

Isolation of inclusion bodies and purification of recombinant HLA-A*01 class I heavy chains.

- 5 Frozen cell preparations (from 200 ml cultures) were ruptured by sonication in 10 ml 20 mM tris, pH 8 with lyzosome (100 µg/ml), EDTA (1 mM), PMSF (50 µg/ml) and incubated 20 min. at room temperature. Subsequently, DNase (10 µg/ml) and MgCl (10 mM) were added. After clearance, the inclusion bodies were partially purified by centrifugation in 15 min. at 10,000 g. Pellets containing inclusion bodies were washed 3 times in the tris
- 10 buffer. The pellets were finally solubilised by a 2 hour incubation in 3 ml 8 M urea, 20 mM tris, pH 8.0 with PMSF and EDTA at 4°C. Insoluble material was removed by centrifugation. The supernatant was harvested and passed over 0.22 µm filters before storage at -80°C. These preparations contained HLA-A*0201 class I heavy chains with a purity about 60 - 80% estimated from SDS-PAGE. The HLA-A*0201 class I heavy chains were purified
- 15 using an anion-exchange (Fast Flow, Pharmacia) column (1 x 25 cm). Preparations with partially purified heavy chains were diluted 5 fold with 8 M urea, 20 mM tris, pH 8.0 and applied the ion-exchange matrix. The column was washed with 20 ml 8 M urea, 20 mM tris, pH 8.0. and proteins were eluted in a gradient of 0 to 500 mM NaCl in 8 M urea, 20 mM tris, pH 8.0 buffer. Eluted proteins were monitored by BCA protein determination,
- 20 SDS-PAGE analysis and peptide binding capacity. Fractions containing highly purified monomer heavy chain proteins and with high capacity of peptide binding were pooled and stored at -80°C.

III.4 Purification of b2m and monoclonal antibodies.

- Recombinant human and mouse b2m was produced and purified as described previously
- 25 (Pedersen et al., 1995). The monoclonal antibodies, W6/32 (α-HLA class I), BB7.2 (α-HLA- HLA-A*0201), 11-4.1 (α-Kk), S13-29 (α-Kk), 28-14-8S (α-H-2D^b), B22-249 (α-H-2D^b), were produced as ascites and purified by protein A chromatography (most of these hybridomas are from ATCC)

Radio-iodination of b2m and of peptide.

- 30 HLA-A*0201 and H-2D^b specific peptides were purified by reverse phase HPLC chromatography and lyophilised. The peptide (1-2 µg) was radiolabelled to a specific activity of 60

mCi/ μ g as previously described (Olsen et al., 1994). The fraction of labeled peptide binding to recombinant or native MHC class I was routinely 80%.

Electrophoresis

- One-dimensional mini-slab SDS-polyacrylamide gel electrophoresis (PAGE) was performed in homogeneous polyacrylamide gels (15%). Samples were boiled in Laemmli sample buffer with or without 50 mM DTT prior to SDS-PAGE analysis. Proteins were stained with Coomassie Blue R-250.

Peptide binding to recombinant HLA-A*201 class I molecules (tracer binding).

- Denatured recombinant HLA-A*0201 heavy chains from the anion exchange purification were tested for ability of peptide binding in the presence of b2m. Binding of peptide to recombinant heavy chains was conducted essentially as a conventional folding by dilution assay (Garboczi et al, 1992) - except that the amount of radiolabelled peptide was in tracer amount i.e. about 2 nM. Typically, 1 microliter of denatured heavy chain solution from fractions of ion-exchange purification was diluted 100 fold in a folding buffer described below.
- The reaction was incubated for at least 4 hours. The peptide binding was examined by Sephadex G25 spun column chromatography (Buus et al., 1995). All experiments have been conducted two or more times. Optimizing the binding of peptides to recombinant H-2D^b and HLA-A*0201 revealed an optimal folding buffer consisting of 20 mM Tris pH 7, 150 mM NaCl, 1 mM EDTA, 50 μ g/ml PMSF, 1 μ M b2m and 1-2 nM tracer peptide. It is noteworthy that classical folding agents such as L-arginine and bacterial chaperonins such as GRO-EL/ES had a negative impact on the peptide binding (data not shown). Normal ranges of GSH/GSSG concentrations e.g. 1.8 mM/0.2 mM had no effect on the peptide interaction. Peptide binding analysis in a temperature range from 4°C to 37°C indicated optimal binding at 18°C. Peptide binding analysis in a range of pH 5.5 to 9 indicated an optimal binding at pH 6.5 - 7.5. Kinetic studies showed that tracer peptide binding equilibrium was established after 4 hours incubation at 18°C.

Generation of functional HLA-A*201 class I molecules (scaled-up folding).

- Denatured and purified HLA-A*201 heavy chain preparations (300 - 600 μ g/ml) were folded by 100 fold dilution in a folding buffer (see above) to which peptide was added to a concentration of about 10 μ M. The reaction was concentrated 10-20 fold at 4°C using Amicon filter with a cut-off of about 10 kD. The concentrate was incubated at 4°C for 1

hour and centrifuged 15 min. at 15,000 g. Supernatants were concentrated further using Centricon units with a cut-off of about 3 kD at 4°C. Centrifugation was repeated and supernatants were applied to size-exclusion chromatography (Sephadex G50) to exclude free b2m and peptide. Fractions containing functional HLA-A*201 class I were pooled and
5 concentrated to a final concentration of about 1 mg/ml.

Results

Expression and purification of denatured recombinant HLA-A*201 heavy chain.

- Recombinant HLA-A*0201 and H-2D^b were expressed and partially purified essentially as described by Garboczi et al. (Garboczi et al., 1992). The cells were induced with 0.4 mM
10 IPTG at a cell density about 0.6 and incubated for 3 hours at 37°C. The electrophoretic mobility of boiled and reduced samples with and without IPTG were analyzed in 15% SDS-PAGE gels. Yields of recombinant HLA-A*0201 were estimated to be about 40-50 mg/L culture corresponding to the predominating protein band of about 33 kD in figure 1, lane 3.
- 15 To isolate the inclusion bodies, the cells were ruptured by sonication. The cells expressing recombinant heavy chains (from a 200 ml culture) were centrifuged and pellets re-solubilised in buffers containing 20 mM tris, pH 8, lysozyme, PMSF and EDTA. Subsequently, DNase and MgCl were added. After clearance of the solution (20-30 min. at 22°C) was centrifuged to pellet the inclusion bodies. Pellet was washed 3 times in tris buffer pH 8
20 and finally resolubilized in 8 M urea and stored at -80°C.

The partially purified proteins from inclusion bodies were further fractionated, using anion ion-exchange chromatography. Reasons for this purification step is to enrich the preparation for recombinant HLA-A*0201 molecules of high folding efficiently and to avoid heterogeneity (minor contaminants, minor enzymatic changes of the heavy chains in the bacteria etc.)
25

- Monomer recombinant HLA-A*0201 and H-2D^b were eluted in a gradient (0-500 mM) NaCl. Recombinant HLA-A*0201 and H-2D^b heavy chain monomers eluted at about 200 mM and 350 mM NaCl, respectively. The purity, concentration and functionality of the purified heavy chains were analyzed in SDS-PAGE (figure 1, lane 4 and figure 2), and by
30 BCA-protein determination assays and by tracer peptide binding analysis (figure 4).

Anion-exchange purification and non-reducing SDS-PAGE analysis showed that majority, if not all, of the recombinant HLA-A*0201 protein, solubilised from isolated inclusion bodies, were oxidized i.e. contained di-sulphide bridges. As shown in figure 2 the eluted recombinant HLA-A*0201 heavy chains migrated as two distinct bands (hereafter called 2a and 3, respectively) in non-reducing SDS-PAGE analysis. We could not separate protein 2a from protein 3 using size-exclusion, ion-exchange or hydrophobic chromatography. Under reducing conditions, the two HLA-A*0201 heavy chains co-migrates slowly as one single protein band-called band 1 (figure 3, compare lane 2 and 3 with lane 4). This band correspond to the dominating HLA-A*0201 protein band in figure 1 (lane 3). In conclusion the proteins appears packed in inclusion bodies with intact di-sulphur bridges leading to at least two distinct monomers. Folding experiments as described below, clearly shows that only protein band 2a folds into a functional recombinant HLA-A*0201 complex (figure 3, compare lane 1 and 2. Protein band 3 misfold and aggregates during a folding by dilution process. Presumably, protein band 2a contain correct di-sulphur bridge(s) whereas protein band 3 is incorrect. Also, chemical cross linking of radiolabelled peptide to recombinant HLA-A*0201 heavy chain identified the protein band 2a as the peptide receptor (data not shown).

The fast migration of proteins packed in inclusion bodies seems to be a general phenomenon. We have observed faster migration of widely different proteins as human b2m, domains of the gamma and epsilon chains of the CD3 complex proteins, H2-K^k, MHC class II Alfa and beta chains in non-reducing SDS-PAGE analysis. Also recombinant H-2D^b molecules migrates with higher speed under non-reducing conditions (data not shown). In comparison with HLA-A*0201 heavy chains, the non-reduced H-2D^b heavy chains migrates as one single fast migrating band which migrates slower in reducing SDS-PAGE analysis (data not shown).

Binding of radiolabelled peptides to partially purified denatured recombinant HLA-A*201 heavy chains.

Recombinant heavy chains fractionated by anion exchange chromatography were tested for their ability to bind radiolabelled specific peptides added during a folding by dilution process (see materials and methods). As shown in (figure 4) there is a good correlation between appearance of monomer recombinant HLA-A*0201 heavy chain monomers (bands 2a and 3) and the ability of peptide binding. Purified heavy chains from urea preparation with reducing agent (more than 0.1 mM DTT) do not bind peptides after bio-

chemical purification even in presence of GSH/GSSG. Thus, the ability of peptide binding is related to heavy chains with pre-formed di-sulphide bridges.

Both recombinant HLA-A*0201 and recombinant H-2D^b heavy chains bound specific radioligand which could be inhibited by specific peptides, but not by non-binding peptides.

- 5 The recombinant HLA-A*0201 in complex with radiolabelled peptides could be precipitated with specific antibodies against HLA-A*0201 but not with antibodies against H2-Kk and H-2D^b molecules, indicating the generation of correct HLA-A*0201 (figure 5).

Binding of radiolabelled peptides to highly purified denatured recombinant HLA-A2*201 heavy chains.

- 10 Generation of functional MHC class I from a denatured state of heavy chains with pre-formed di-sulphur bridges was evaluated using radiolabelled peptide and radiolabelled b2m. Fully purified HLA-A*0201 heavy chains corresponding to protein band 2a were obtained through denaturation of functional recombinant HLA-A*0201 using 8 M urea. The denatured proteins were fractionated by size exclusion chromatography (Sephadex G50)
- 15 in a 20 mM tris buffer pH 8 with 8 M urea and the heavy chain was harvested in the void volume.

Binding of radiolabelled ligands (peptide or b2m) were done by dilution in folding buffer as described above. A dose response (figure 6) shows very efficient and sensitive binding of radiolabelled peptide to the denatured heavy chain during the folding process. In compari-

- 20 son, conventional affinity purified MHC class I molecules requires 10 to 50 fold higher concentration to bind similar amounts of peptide.

The high efficiency of peptide binding was analyzed in inhibition assay and by Scatchard analysis. As shown in figure 7, the recombinant HLA-A*0201 only interact with specific peptides. The Scatchard analysis (figure 7, insert) revealed a simple straight Scatchard

- 25 plot and an affinity equilibrium constant about 30 nM. Importantly, the fraction of active receptor was about 80 -90 % of the input protein. Thus, the HLA-A*201 heavy chain was fully active without being preoccupied by peptides from the bacteria or derived from any of the subsequent handling steps. In comparison, conventional affinity purified MHC class I preparations are preoccupied with peptide having a limited number of active receptors,
- 30 typically 2%.

The peptide binding was very much dependent on b2m. As shown in figure 8, increasing doses of b2m added to the binding reaction facilitates binding of radiolabelled peptide. Ab-

sence of b2m completely prevented peptide binding to the heavy chain. Also the reverse reaction i.e. b2m binding to heavy chain show some dependence on the presence of specific peptides (figure 9). The peptide is however not an absolute requirement as b2m does bind, albeit with a lower affinity, to the heavy chain in the absence of peptide.

- 5 The effect of peptides on b2m interaction was further analyzed in kinetic studies. Dissociation rates of HLA-A*201 complexes consisting of radiolabelled b2m and heavy chains in the presence or absence of specific peptides were analyzed. Hetero-dimer complexes i.e. generated and maintained in absence of specific peptides dissociated rapidly with a half-life about 4 hours at 37°C. Trimeric peptide-b2m- heavy chain complexes i.e. generated
10 and maintained in presence of peptides were in comparison stable with a half-life about 14 hours. Thus peptides stabilizes the b2m binding. We conclude that a HLA-A*201 complex is generated through a primary interaction between the denatured heavy chain and b2m. The interaction generates a hetero-dimer, which express a high affine peptide binding site. Peptides in their turn increase the affinity of bound b2m molecules resulting in
15 stable functional HLA-A*201 complexes.

Generation of functional HLA-A*0201.

- Fractions with high capacity of peptide binding (about 15 ml corresponding to 60-70% of the fractions with monomer heavy chain) were harvested and pooled for generation (folding) of functional HLA-A*0201 molecules. Recombinant HLA-A*0201 heavy chains were
20 diluted in folding buffer as described above. 500 µg, in 1 ml, partially purified heavy chain (corresponding to about 13 ml bacterial culture) was added to 99 ml buffer and immediately concentrated using Amicon filters with a cut off about 10 kD. 5-10 ml concentrate (obtained within 1 hour) was incubated for 1 hour at 4°C before centrifugation at 15,000g to remove aggregates. The supernatant was harvested and further concentrated to about
25 150-200 µl using centricon units with cut off about 3 kD. After additional centrifugation the folded HLA-A*0201 was purified using size exclusion chromatography (G50) which retain free b2m and peptides. Fractions containing the assembled HLA-A*0201 were pooled and concentrated using centricon to a final concentration about 1 mg/ml. The folding efficiency was about 40 - 50% calculated from the amount of added denatured protein. The yield of
30 functional HLA-A*0201, through the entire process correspond to about 10 mg functional HLA-A*0201 /L bacterial culture. The whole process of folding and purification can be conducted within 24 hours. Notice that folding was without addition of conventional agents as L-arginine and GSH/GSSG. The former inhibits folding of denatured HLA-A*0201

heavy chains with pre-formed disulfide bridges. The latter do not effect the outcome. The folded recombinant HLA-A*0201 molecules have routinely been tested in SDS-PAGE (figure 3) and for reactivity with specific antibodies (figure 5). Recombinant H-2D^b complexes generated by this procedure using biotinylated b2m was recently further assembled in oligomeric complexes using streptavidin. The oligomerized ("tetramers") H-2D^b complexes was used for FACS staining of specific T cells (figure 11) and for staining of T cells as assessed by confocal microscopy. The latter demonstrated specific binding and internalisation.

10 REFERENCES

- Altman, J. D., Moss, P. A. H., Goulder, P. J. R., Barouch, D. H., McHeyzer-Williams, M. G., Bell, J. I., McMichael, A. J., and Davis, M. M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274, 94-6.
- 15 Björkman, P. J., Saper, M. A., Samaoui, B., Bennett, W. S., Strominger, J. L., and Wiley, D. C. (1987). Structure of the human class I histocompatibility antigen, HLA- A2. *Nature* 329, 506-512.
- 20 Buus, S., Sette, A., Colon, S. M., and Grey, H. M. (1988). Autologous peptides constitutively occupy the antigen binding site on Ia. *Science* 242, 1045-1047.
- Buus, S., Sette, A., Colon, S. M., Jenis, D. M., and Grey, H. M. (1986). Isolation and characterisation of antigen-Ia complexes involved in T cell recognition. *Cell* 47, 1071-1077.
- 25 Buus, S., Sette, A., Colon, S. M., Miles, C., and Grey, H. M. (1987). The relation between major histocompatibility complex restriction and the capacity of Ia to bind immunogenic peptides. *Science* 235, 1353-1358.
- 30 Buus, S., Stryhn, A., Winther, K., Kirkby, N., and Pedersen, L. Ø. (1995). Receptor-ligand interactions measured by an improved spun column chromatography technique. A high efficiency and high throughput size separation method. *Biochim. Biophys. Acta* 1243, 453-460.

- Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H.-G. (1991). Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 351, 290-296.
- 5 Gallimore, A., Glithero, A., Godkin, A., Tissot, A. C., Pluckthun, A., Elliott, T., Hengartner, H., and Zinkernagel, R. (1998). Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic T lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes. *Journal of Experimental Medicine* 187, 1383-93.
- 10 Garboczi, D. N., Hung, D. T., and Wiley, D. C. (1992). HLA-A2-peptide complexes: Refolding and crystallization of molecules expressed in *Escherichia coli* and complexed with single antigenic peptides. *Proceedings of the National Academy of Sciences (USA)* 89, 3429-3433.
- 15 Henderson, R. A., Michel, H., Sakaguchi, K., Shabanowitz, J., Appella, E., Hunt, D. F., Engelhard, V. H. (1992). HLA-A2.1 associated peptides from a mutant cell line: A second pathway of antigen presentation., *Science* 255, 1264-66
- 20 Kast, W. M., Brandt, R. M. P., Sidney, J., Drijfhout, J.-W., Kubo, R. T., Grey, H. M., Melief, C. J. M., and Sette, A. (1994). Role of HLA-A motifs in identification of potential CTL epitopes in human papillomavirus type 16 E6 and E7 proteins. *Journal of Immunology* 152, 3904-3912.
- 25 Matsumura, M., Fremont, D. H., Peterson, P. A., and Wilson, I. A. (1992). Emerging principles for the recognition of peptide antigens by MHC class I molecules. *Science* 257, 927-934.
- 30 Matsumura, M., Saito, Y., Jackson, M. J., Song, E. S., Peterson, P. A., (1992), In vitro peptide binding to soluble empty class I major histocompatibility complex molecules isolated from transfected *Drosophila melanogaster* cells, *Journal of Biological Chemistry*, 257, 23589-96
- Olsen, A. C., Pedersen, L. Ø., Hansen, A. S., Nissen, M. H., Olsen, M., Hansen, P. R.,
35 Holm, A., and Buus, S. (1994). A quantitative assay to measure the interaction between

- immunogenic peptides and MHC class I molecules. *European Journal of Immunology* 24, 385-392.
- Parker, K. C., Bednarek, M. A., and Coligan, J. E. (1994). Scheme for ranking potential
5 HLA-A2 binding peptides based on independent binding of individual peptide side-chains. *Journal of Immunology* 152, 163-175.
- Parker, K. C., Carreno, B. M., Sestak, L., Utz, U., Biddison, W. E., and Coligan, J. E. (1992). Peptide binding to HLA-A2 and HLA-B27 isolated from *Escherichia coli*. *Journal of*
10 *Biological Chemistry* 267, 5451-5459.
- Parker, K. C., Silver, M. L., and Wiley, D. C. (1992). An HLA-A2/beta 2-microglobulin/peptide complex assembled from subunits expressed separately in *Escherichia coli*. *Mol Immunol* 29, 371-8.
15
- Parker, K. C., and Wiley, D. C. (1989). Overexpression of native human beta 2-microglobulin in *Escherichia coli* and its purification. *Gene* 83, 117-24.
- Parker, K. C., and Wiley, D. C. (1990). Reconstitution of HLA-A2 from subunits expressed
20 separately in *Escherichia coli* requires antigenic peptide. ?, 119.
- Pedersen, L. Ø., Stryhn, A., Holtet, T. L., Etzerodt, M., Gerwien, J., Nissen, M. H., Thøgersen, H. C., and Buus, S. (1995). The interaction of beta 2-microglobulin (b₂m) with mouse class I major histocompatibility antigens and its ability to support peptide binding.
25 A comparison of human and mouse b₂m. *Eur. J. Immunol.* 25, 1609-1616.
- Rupert, J., Sidney, J., Celis, E., Kubo, R. T., and Sette, A. (1993). Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. *Cell* 74, 929-937.
- 30 Sambrook, Fritsch and Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989
- Sette, A., Buus, S., Colon, S. M., Smith, J. A., Miles, C., and Grey, H. M. (1987). Structural characteristics of an antigen required for its interaction with Ia and recognition by T
35 cells. *Nature* 328, 395-399.

Stryhn, A., Pedersen, L. Ø., Romme, T., Holm, C. B., Holm, A., and Buus, S. (1996). Peptide binding specificity of major histocompatibility complex class I resolved into an array of apparently independent sub-specificities: quantitation by peptide libraries and improved
5 prediction of binding. *European Journal of Immunology* 26, 1911-18.

Vierboom, M. P., Nijman, H. W., Offringa, R., van der Voort, E. I., van Hall, T., van den Broek, L., Fleuren, G. J., Kenemans, P., Kast, W. M., and Melief, C. J. (1997). Tumor eradication by wild-type p53-specific cytotoxic T lymphocytes. *Journal of Experimental*
10 *Medicine* 186, 695-704.

Walter, J. B., Garboczi, D. N., Fan, Q. R., Zhou, X., Walker, B. D., and Eisen, H. N. (1998). A mutant human beta2-microglobulin can be used to generate diverse multimeric class I peptide complexes as specific probes for T cell receptors. *J Immunol Methods* 214,
15 41-50.

Wei, M. L., Cresswell, P. (1992), HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence derived peptides. *Nature* 356, 443-446.

20 12th International Histocompatibility Workshop Cell Lines Panel Database ("HLA: Genetic diversity of HLA. Functional and Medical Implication", Ed. Dominique Charron, EDK Press, 1997)

CLAIMS

1. A process of producing a functional immunoglobulin superfamily protein, which has at least one disulphide bond when functional, the process comprising the steps of
- 5
- (i) providing a bacterial cell comprising a gene coding for the protein, the gene is expressible in said cell,
 - (ii) cultivating the cell under conditions where the gene is expressed,
 - 10 (iii) isolating the protein from the cell without reducing it,
 - (iv) subjecting the isolated protein to a folding treatment.
- 15 2. A process of producing a plurality of functional proteins including at least one from the immunoglobulin superfamily and where the plurality of functional proteins when functional has at least one intramolecular and/or one intermolecular disulphide bond, the process comprising the steps of
- 20
- (i) providing a bacterial cell comprising a plurality of genes coding for one protein each, all genes being expressible in said cell,
 - (ii) cultivating the cell under conditions where the genes are expressed,
 - 25 (iii) isolating the proteins from the cell without reducing them,
 - (iv) subjecting the isolated proteins to a folding treatment.
3. A process of producing a functional immunoglobulin superfamily protein, which has at least one disulphide bond when functional, the process comprising the steps of
- 30
- (i) providing a cell comprising a gene coding for the protein, the gene is expressible in said cell, the protein being expressed as an aggregate,
 - 35 (ii) cultivating the cell under conditions where the gene is expressed,

- (iii) isolating the protein aggregate from the cell without reducing it,
- (iv) subjecting the isolated protein to a folding treatment.

5

4. A process according to any of claims 1-3 wherein the yield of functional protein produced according to the process relatively to the yield of functional protein obtained under essentially similar conditions but where step (iii) is performed under reducing conditions, is increased by at least 10%, such as at least 20%, at least 40%, at least 50%, at least 70%, or at least 100%.

10

5. A process according to any of claims 1-4 wherein when step (iii) is performed under non-reducing conditions, the speed of the process compared to when step (iii) is performed under reducing conditions is at least 10% faster.

15

6. A process according to any of claims 1-5 wherein the purity of the functional protein produced according to the process relatively to the purity of the resulting functional protein obtained under essentially similar conditions but where step (iii) is performed under reducing conditions, is increased by at least 10%.

20

7. A process according to any of claims 1-6 wherein the protein is an immunoglobulin superfamily protein selected from the group consisting of antibodies, immunoglobulin variable (V) regions, immunoglobulin constant (C) regions, immunoglobulin light chains, immunoglobulin heavy chains, CD1, CD2, CD3, Class I and Class II histocompatibility molecules, β_2 microglobulin (β_2m), lymphocyte function associated antigen-3 (LFA-3) and Fc γ RIII, CD7, CD8, Thy-1 and Tp44 (CD28), T cell receptor, CD4, polyimmunoglobulin receptor, neuronal cell adhesion molecule (NCAM), myelin associated glycoprotein (MAG), P myelin protein, carcinoembryonic antigen (CEA), platelet derived growth factor receptor (PDGFR), colony stimulating factor-1 receptor, $\alpha\beta$ -glycoprotein, ICAM (intercellular adhesion molecule), platelet and interleukins.

25

8. A process according to any of claims 1-7 wherein the immunoglobulin superfamily protein is a vertebrate, e.g. protein such as a human, a murine, a rat, a porcine, a bovine, or an avian protein.

35

9. A process according to any of claims 1-8 wherein the immunoglobulin superfamily protein is a MHC.

10. A process according to claim 9 wherein the MHC protein is a human MHC.

5

11. A process according to claim 9 or 10 wherein the MHC protein is a MHC class I protein selected from the group consisting of a heavy chain, a heavy chain combined with a β_2m , and a functional mature MHC class I protein; or a MHC class II protein selected from the group consisting of an α/β dimer and an α/β dimer with a peptide.

10

12. A process according to any of claims 9-11 wherein the produced MHC protein is obtained as a peptide free MHC protein.

13. A process according to any of claims 7-12 whereby when the folding process is finalized at least 25% of the produced immunoglobulin superfamily protein is obtained in a functional form.

15

14. A process according to any of claims 1-13 wherein the protein in step (ii) is produced as inclusion bodies.

20

15. A process according to any of claims 1-14 wherein step (iii) is performed under non-reducing conditions without altering the redox state.

16. A process according to any of claims 1-15 wherein the process further comprises a step wherein the isolated protein from step (iii) is subjected to a purification step before step (iv).

25

17. A process according to any of claims 1-16 wherein the folding treatment (iv) is performed in an aqueous medium and at least one buffer compound.

30

18. A process according to any of claims 1-17 wherein the folding treatment is performed essentially in the absence of reducing agents, such as DTT.

19. A process according to any of claims 1-18 wherein the expressed protein is located intracellularly.

35

20. A process according to any of claims 1-19 wherein the expressed protein is located periplasmatically.
- 5 21. A process according to any of claims 1-20 wherein the expressed protein is translocated extracellularly.
22. A process according to any of claims 1-21 wherein the protein is expressed in a glycosylated form.
- 10 23. A process according to any of claims 1-22 wherein the protein is expressed in a phosphorylated form.
24. A process according to any of claims 1-23 wherein the protein is glycosylated or
15 phosphorylated *in vitro*.
25. A process according to any of claims 1-24 wherein the protein comprises no unpaired Cysteine residues.
- 20 26. A process according to any of claims 1-25 wherein the protein comprises 1 unpaired Cysteine residue.
27. A process according to any of claims 1-26 wherein the protein comprises at least 2, such as at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least
25 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or at least 20 Cysteine residues.
28. A process according to any of claims 1-27 wherein the protein comprises an even number of Cysteine residues.
- 30 29. A process according to any of claims 1-28 wherein the protein is having at most 20, such as at most 14, at most 10, at most 8, at most 5, at most 4, at most 3, or at most 2 Cysteine residues.